

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:

A61K 38/00, C07K 1/00, 5/00, 7/00,  
14/00, 17/00

A1

(11) International Publication Number:

WO 95/07707

(43) International Publication Date:

23 March 1995 (23.03.95)

(21) International Application Number:

PCT/US94/10368

(22) International Filing Date:

14 September 1994 (14.09.94)

(30) Priority Data:

08/121,101

14 September 1993 (14.09.93)

US

(71) Applicant: CYTEL CORPORATION [US/US]; 3523 John  
Hopkins Court, San Diego, CA 92121 (US).

(72) Inventors: SETTE, Alessandro; 5531 Beaumont Avenue, La  
Jolla, CA 92037 (US). GAETA, Federico, C., A.; 3226  
Rim Rock Circle, Olivenhain, CA 92024 (US). GREY,  
Howard, M.; 8307 Caminito Helecho, La Jolla, CA 92037  
(US). SIDNEY, John; 8541-D Villa La Jolla Drive, La Jolla,  
CA 92037 (US). ALEXANDER, Jerry, L.; 117 Mangano  
Circle, Encinitas, CA 92024 (US).

(74) Agents: BASTIAN, Kevin, L. et al.; Townsend and Townsend  
Khourie and Crew, Steuart Street Tower, 20th floor, One  
Market Plaza, San Francisco, CA 94105-1492 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH,  
CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG,  
KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW,  
NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT,  
UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES,  
FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD,  
TG), ARIPO patent (KE, MW, SD).

Published

With international search report.

Before the expiration of the time limit for amending the  
claims and to be republished in the event of the receipt of  
amendments.

(54) Title: ALTERATION OF IMMUNE RESPONSE USING PAN DR-BINDING PEPTIDES

(57) Abstract

The present invention provides compositions and methods of inhibiting or inducing activation of T cells in a patient. The methods comprise administering a therapeutically effective dose of pharmaceutical compositions comprising a pharmaceutically acceptable carrier and peptides of between about 4 and about 20 residues, that bind antigen binding sites on MHC molecules encoded by substantially all alleles of a DR locus. These peptides are referred to as pan DR binding peptides. The pan DR binding peptides can be used to inhibit immune responses associated with immunopathologies, such as autoimmunity, allograft rejection and allergic responses. The peptides can also be used in combination with CTL peptides to enhance a CTL response.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

5

## ALTERATION OF IMMUNE RESPONSE USING PAN DR-BINDING PEPTIDES

### BACKGROUND OF THE INVENTION

10 This application is a continuation-in-part of pending application United States Serial No. 08/121,101 filed September, 14, 1993 which is incorporated herein by reference.

The present invention relates to compositions and methods for preventing, treating or diagnosing a number of pathological states such as autoimmune diseases, viral  
15 diseases and cancers. In particular, it provides novel peptides capable of binding selected major histocompatibility complex (MHC) molecules and either inducing or inhibiting an immune response.

MHC molecules are classified as either Class I or  
20 Class II molecules. Class II MHC molecules are expressed by specialized antigen presenting cells (APC) such as macrophages, dendritic cells, or B cells. The Class II MHC molecules usually associate with peptide fragments derived from processing of protein antigens which enter the endocytic  
25 pathway from the APC exterior. The MHC-peptide complexes are subsequently presented for scrutiny to CD4+ T helper cells which are then activated, proliferate and amplify the immune response to the particular immunogenic peptide that is displayed. Activation of T cells requires engagement of the T  
30 Cell Receptor (TCR) by its ligand, a bi-molecular complex of an MHC molecule and a peptide antigen (Shimonkevitz, et al., *J. Immunol.* 133, 2067-2074 (1984); Babbitt, et al., *Nature* 317, 359-361 (1985); Buus, et al., *Cell* 47, 1071-1077 (1986); Townsend, A., and Bodmer, H., *Annu. Rev. Immunol.* 7, 601-624.

35 Inappropriate activation of T cells is a component of a number of immunopathologies, such as autoimmunity, allograft rejection and allergic responses. Exemplary autoimmune diseases include rheumatoid arthritis, multiple sclerosis, and myasthenia gravis. Allergic responses  
40 involving T cell activation include allergies to various

pollens, dust mites and the like. In addition, foreign infectious diseases may cause immunopathology (e.g., Lyme disease, hepatitis, LCMV, post-streptococcal endocarditis, or glomerulonephritis). Food hypersensitivities, such as celiac disease and Crohn's disease, as well as other allergic diseases, have been associated with particular MHC alleles or suspected of having an autoimmune component.

The most commonly used approach to treating these conditions is to suppress the immune system, typically by using immunosuppressive drugs. Another approach has been proposed for cases in which the MHC allele associated with the condition is known, involving selective blockade of a given MHC allele. However, where a number of MHC restrictions are involved, approaches other than selective blockade must be found.

Immunochemical studies of the requirements for peptide binding to class II molecules have been carried out. The binding motifs of several murine and human class II MHC alleles have been defined, and motif analysis by sequencing of naturally processed peptides has also recently been described for various class II types (Rudensky et al., *Nature* 353, 622-627 (1991); Chicz et al., *Nature* 358, 764-768 (1992); Hunt et al., *Science* 256, 1817-1820 (1992); Rudensky et al., *Nature* 359, 429-431 (1992)).

In the case of DR molecules in particular, it has been shown (Brown et al., *Nature* 364, 33-39 (1993)) that a large hydrophobic anchor engaging a corresponding hydrophobic pocket of the MHC binding groove is the most crucial determinant of peptide-DR interactions. Several other anchors play definite, albeit less prominent roles and help determine allelic specificity. Recently it has also been emphasized that the peptide backbone of the C-terminal half of the peptide molecule is engaged in direct hydrogen bonding with the walls of the MHC binding groove (Krieger et al., *J. Immunol.* 146, 2331-2340 (1991)); O'Sullivan et al., *J. Immunol.* 146, 1240-1246 (1991); and O'Sullivan et al., *J. Immunol.* 147, 2663-2669 (1991).

Although allele-specific polymorphic residues that line the peptide binding pockets of MHC alleles tend to endow each allele with the capacity to bind a unique set of peptides, there are many instances in which a given peptide has been shown to bind to more than one MHC specificity. This has been best documented in the case of the human DR isotype, in which it has been noted that several DR alleles appear to recognize similar motifs, and independently, several investigators reported degenerate binding and/or recognition of certain epitopes in the context of multiple DR types, leading to the concept that certain peptides might represent "universal" epitopes (Busch et al., *Int. Immunol.* 2, 443-451 (1990); Panina-Bordignon et al., *Eur. J. Immunol.* 19, 2237-2242 (1989); Sinigaglia et al., *Nature* 336, 778-780 (1988); O'Sullivan et al., *J. Immunol.* 147, 2663-2669 (1991) Roache et al., *J. Immunol.* 144, 1849-1856 (1991); Hill et al., *J. Immunol.* 147, 189-197 (1991)). However, although the previously reported epitopes do have the capacity to bind to several DR alleles, they are by no means universal.

Accordingly, the present invention provides DR binding peptides, called "pan DR binding peptides" that are recognized by a broad pattern of DR alleles. According to the present invention such Pan DR binding peptides may be used as potent immunogens for class II restricted T cells, and as a useful human peptide-based immunosuppressant, vaccine or therapeutic agent.

#### SUMMARY OF THE INVENTION

The present invention provides compositions and methods of inhibiting or inducing activation of T cells in a patient. The methods comprise administering a therapeutically effective dose of pharmaceutical compositions comprising a pharmaceutically acceptable carrier and peptides of between about 4 and about 20 residues, that bind antigen binding sites on MHC molecules encoded by substantially all alleles of a DR locus. These peptides are referred to as pan DR binding peptides. The pan DR binding peptides can be used to inhibit

immune responses associated with immunopathologies, such as autoimmunity, allograft rejection and allergic responses.

When used to enhance an immune response, the pan DR peptide can act as a T helper peptide and be administered with a CTL inducing peptide. The T helper peptide may be linked to the CTL inducing peptide to form a CTL/T helper peptide conjugate. The conjugates may also be further modified. For instance, the conjugate may be acetylated, palmitylated, or acylated with a fatty acid, or linked to a carrier. The CTL peptide may also be linked to the T helper peptide by a spacer molecule, such as Ala-Ala-Ala. Alternatively, pan DR peptides can be used to augment antibody responses. As with the induction of CTL responses, pan DR peptides can be, administered either linked to, or admixed with, antibody inducing determinants.

Pan DR peptides can be described using various conventions. For example, preferred pan DR peptides have the formula  $R_1-R_2-R_3-R_4-R_5$ , proceeding in the direction from the amino-terminus of the peptide ( $R_1$ ) to the carboxy-terminus ( $R_5$ ), where  $R_1$  is a D-amino acid followed by alanine or lysine;  $R_2$  is cyclohexylalanine, tyrosine, or phenylalanine;  $R_3$  is 3 or 4 amino acids each of which is independently selected from the group consisting of alanine, isoleucine, serine and valine;  $R_4$  is threonine-leucine-lysine, lysine-theronine, or tryptophan-threonine-leucine-lysine; and  $R_5$  consists of 2 to 4 amino acids followed by a D-amino acid, where each of the 2 or 4 amino acids is independently selected from the group consisting of alanine, serine and valine. According to this formula, more preferred pan DR peptides have the formula  $R_1-R_2-R_3-R_4-R_5$ , where  $R_1$  is D-alanine followed by alanine or lysine;  $R_2$  is cyclohexylalanine or phenylalanine;  $R_3$  is 3 or 4 amino acids each of which is selected from the group comprising alanine, isoleucine, and valine;  $R_4$  is threonine-leucine-lysine, lysine-theronine, or tryptophan-threonine-leucine-lysine; and  $R_5$  is 2 to 4 alanines followed by D-alanine.

Pan DR peptides can also be described using the one-letter code for amino acids commonly found in proteins and

specifying a designation for D-amino acids or unusual amino acids. Using this convention, preferred pan DR peptides of the invention include the following: oZXZZZKTZZZZo, oZXZZZZTLKZZo, oZXVZZZTLKZZo, oZXIZZZTLKZZo, oKXVZZWTLKZZo, and oKFVZZWTLKZZo wherein o is a D-amino acid, Z is alanine, serine or valine, K is lysine, T is threonine, L is leucine, W is tryptophan, and X is cyclohexylalanine, tyrosine or phenylalanine. Most preferred pan DR peptides include aAXAAAKTAAAAa, aAXAAAATLKAAAa, aAXVAAATLKAAAa, aAXIAAATLKAAAa, aKXVAAWTLKAAAa, and aKFVAAWTLKAAAa wherein a is D-alanine, A is alanine, X is cyclohexylalanine, K is lysine, T is threonine, L is leucine, V is valine, I is isoleucine, W is tryptophan.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1F show representative responses for three of twelve different donors. Antigen specific T cell responses from human PBMC T cell lines generated on day 0 by addition of either peptide 965.10 (closed square), 906.09 (open square), 760.50 (closed circle), or Tetanus toxoid 830-843 (open circle) as assayed on day 14 (second stimulation, Figures 1A, 1B, 1C) and day 28 (third stimulation, Figures 1D, 1E, 1F). A representative of two independent experiments is shown.

Figures 2A and 2B show summaries of antigen specific T cell responses from human PBMC. Figure 2A shows results of a second stimulation, and Figure 2B shows results of a third stimulation. The maximum  $\Delta$  cpm obtained is plotted on the ordinate.

Figures 3A-3F show in vivo immunogenicity of various peptide epitopes as measured by proliferative capacity of primed murine lymph node T cells. C57BL/6J mice were injected with 20  $\mu$ g/mouse (open triangle), 1  $\mu$ g/mouse (closed square), 50 ng/mouse (open square), 2.5 ng/mouse (closed circle), or 0.125 ng/mouse (open circle) of TT 830-843 (Figure 3A), Ova 323-336 (Figure 3B), HBV<sub>c</sub> 128-140 (Figure 3C), 965.10 (Figure 3D), 1024.03 (Figure 3E), and 760.50 (Figure 3F). Ten days

later, draining lymph nodes were removed and T cell proliferation assays performed as described in Example 9. A representative of two independent experiments is shown.

5

#### DEFINITIONS

An oligopeptide or peptide as used herein refers to a chain of at least four amino acid or amino acid mimetics, preferably at least six, more preferably eight to ten, sometimes eleven to fourteen residues, and usually fewer than about thirty residues, more usually fewer than about twenty-five, and preferably fewer than fifteen, e.g., eight to fourteen residues. The oligopeptides or peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

When referring to an amino acid residue in a peptide, oligopeptide or protein the terms "amino acid residue", "amino acid" and "residue" are used interchangeably and, as used herein, mean an amino acid or amino acid mimetic joined covalently to at least one other amino acid or amino acid mimetic through an amide bond or amide bond mimetic.

As used herein, the term "amino acid", when unqualified, refers to an "L-amino acid" or L-amino acid mimetic.

Although the peptides will preferably be substantially free of other naturally occurring proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles.

By biological activity is meant the ability to bind an appropriate MHC molecule and, in the case of peptides useful for stimulating CTL responses, induce a T helper response, which in turn helps induce a CTL response against a target antigen or antigen mimetic. In the case of a peptide



analog antagonist, the analog will have biological activity if it competes with the native peptide for binding to the MHC molecule and thus substantially reduces the ability of the native peptide to stimulate a T cell response.

5 A "pan DR-binding peptide" of the invention is a peptide capable of binding at least about 7 of the 12 most common DR alleles (DR1, 2w2b, 2w2a, 3, 4w4, 4w14, 5, 7, 52a, 52b, 52c, and 53) with high affinity. "High affinity" is defined here as binding with an IC<sub>50</sub> of less than 300nM.

10 Throughout this disclosure, results are expressed in terms of IC<sub>50</sub>'s. Given the conditions in which the assays are run (i.e., limiting MHC and labeled peptide concentrations), these values approximate K<sub>D</sub> values. It should be noted that IC<sub>50</sub> values can change, often dramatically, if the assay  
15 conditions are varied, and depending on the particular reagents used (e.g., MHC preparation, etc.). For example, excessive concentrations of MHC will increase the apparent measured IC<sub>50</sub> of a given ligand.

An alternative way of expressing the binding data, to avoid these uncertainties, is as a relative value to a  
20 reference peptide. The reference peptide is included in every assay. As a particular assay becomes more, or less, sensitive, the IC<sub>50</sub>'s of the peptides tested may change somewhat. However, the binding relative to the reference  
25 peptide will not change. For example, in an assay run under conditions such that the IC<sub>50</sub> of the reference peptide increases 10-fold, all IC<sub>50</sub> values will also shift approximately 10-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak,  
30 or negative binder should be based on it's IC<sub>50</sub>, relative to the IC<sub>50</sub> of the standard peptide.

If the IC<sub>50</sub> of the standard peptide measured in a particular assay is different from that reported in Table I,

TABLE I

Allele	Assay standard	Sequence	Avg. IC50 nM
DR1	HA 307-319	PKYVKONTLKLAT	5
DR2w2b	MBP 78-101	GRTQDENPVVWHFFKNIVTPRTPPP	9.1
DR3	MT 65 kd 3-13	YKTIAFDEEARR	250
DR4w4	HA 307-319	PKYVKONTLKLAT	45
DR4w14	717.01 combinatorial	YARFOSQTTLKOKT	50
DR5	Tet Tox 830-843	OYIKANSKFIGITE	20
DR7	Tet Tox 830-843	OYIKANSKFIGITE	25
DR52a	Tet Tox 1272-1284	NGQIGNDPNRDIL	470
DRw53	717.01 combinatorial	YARFOSQTTLKOKT	58
Dr2w2a	Tet Tox 830-843	OYIKANSKFIGITE	20
DQ3.1	ROIV	YAHAAHAAHAAHAAHAA	15
IAb	ROIV	YAHAAHAAHAAHAAHAA	28
IAd	Ova 323-326	ISOAVHAAHAEINE	110
IEd	lambda rep 12-26	YLEDARRLKAIYEKKK	170
IAs	ROIV	YAHAAHAAHAAHAAHAA	54
IAk	HEL 46-61	YNTDGGSTDYGILOINSR	20
IEk	lambda rep 12-26	YLEDARRLKAIYEKKK	28

then it should be understood that the threshold values used to determine good, intermediate, weak, and negative binders should be modified by a corresponding factor.

5 A "CTL inducing peptide" of the present invention is one derived from selected epitopic regions of potential target antigens, such as tumor associated antigens, including, but not limited to, renal cell carcinoma, breast cancer, carcinoembryonic antigens, melanoma (MAGE-1) antigens, and prostate cancer specific antigen, hepatitis C antigens, 10 Epstein-Barr virus antigens, HIV-1 and HIV-2 antigens, and papilloma virus antigens.

The phrases "isolated" or biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the peptides of the present invention do not 15 contain materials normally associated with their *in situ* environment, e.g., MHC Class I molecules on antigen presenting cells. Even where a protein has been isolated to a homogeneous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the 20 desired protein. Isolated peptides of this invention do not contain such endogenous co-purified protein.

The term "T cell clone" refers to a group of T cells that are progeny of a single virgin lymphocyte and expressing 25 identical immunoglobulin or T cell receptor proteins. The term "virgin" lymphocyte is used here as it is used in Stites et al. *Basic and Clinical Immunology*, 8th Edition, Prentice Hall, Englewood Cliffs, New Jersey (1994) which is incorporated herein by reference.

30 A "T helper peptide" as used herein refers to a peptide recognized by the T cell receptor of T helper cells. The T helper peptides of the present invention are pan DR binding peptides.

35

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

In one embodiment, the present invention relates to the use of pan DR peptides to block an immune response by preventing activation of helper T cells. Because of their

degenerate class II binding capacity, the Pan DR binding peptides may be used as therapeutics in the inhibition of T cell mediated events involved in allograft rejection, allergic responses, or autoimmunity.

5 Alternatively, the pan DR peptides are useful as an adjuvant component in any vaccine formulation to enhance an immune response against an administered immunogen. For instance, the pan DR binding peptides are administered with CTL-inducing peptides to induce a CTL response against, e.g.,  
10 virally infected cells. Alternatively, the pan DR binding peptides are conjugated with a CTL-inducing peptide and administered to induce a CTL response. In another embodiment, the pan DR peptides are conjugated with antibody inducing peptides or admixed with an antibody inducing peptide. The use  
15 of helper peptides to enhance antibody responses against particular determinants is described for instance in Hervas-Stubbs et al. Vaccine 12:867-871 (1994).

The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is  
20 presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is  
25 represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids having D-forms is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as  
30 "Gly" or G.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various  
35 automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, *Solid Phase Peptide Synthesis*, 2d. ed., Pierce Chemical Co. (1984), *supra*.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982), which is incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., *J. Am. Chem. Soc.* 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

#### PAN DR-BINDING PEPTIDES

The present invention provides methods useful for identification of modifications to a starting peptide which

broaden its specificity. For instance, International Application WO 92/02543, which is incorporated herein by reference, describes methods suitable for identifying peptides capable of binding DR molecules. This application describes the use of hemagglutinin from the influenza virus ("HA"), as the source of peptides specifically reacting with HLA-DR. Portions of the protein are screened for reactivity to provide sequences which bind the appropriate DR molecule, such as DR1, DR4w4 or DR4w14.

Once an antigen or peptide thereof which binds to the selected MHC molecule is identified, a "core binding region" of the antigen or peptide may be determined by, e.g. synthesizing overlapping peptides, and/or employing N-terminal or C-terminal deletions (truncations) or additions. In the determination of a core binding region and critical contact residues, a series of peptides with single amino acid substitutions may be employed to determine the effect of electrostatic charge, hydrophobicity, etc. on binding.

Within the core region, "critical contact sites," i.e., those residues (or their functional equivalents) which must be present in the peptide so as to retain the ability to bind an MHC molecule and inhibit the presentation to the T cell, may be identified by single amino acid substitutions, deletions, or insertions. In addition, one may also carry out a systematic scan with a specific amino acid (e.g., Ala) to probe the contributions made by the side chains of critical contact residues.

Peptides of the invention which are relatively insensitive to single amino acid substitutions with neutral amino acids, except at essential contact sites, have been found to tolerate multiple substitutions. Particularly preferred multiple substitutions are small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased

binding affinity for an MHC molecule may also be achieved by such substitutions, compared to the affinity of the parent peptide. In any event, such "spacer" substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

The effect of single amino acid substitutions may also be probed using D-amino acids. Such substitutions may be made using well known peptide synthesis procedures, as described in e.g., Merrifield, Science 232:341-347 (1986), Barany and Merrifield, The Peptides, Gross and Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, Solid Phase Peptide Synthesis, (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein.

The peptides employed in the subject invention need not be identical to peptides disclosed in the Example section, below, or to a particular antigenic protein sequence of CTL peptides, so long as the subject compounds are able to bind to the appropriate MHC molecules or provide for cytotoxic T lymphocytic activity against the target antigenic protein. Thus, one of skill will recognize that a number of conservative substitutions can be made without substantially affecting the activity of the peptide. Conservative substitutions are those involving replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another.

Using the general approach described above, a binding motif for a particular MHC allele can be determined. To broaden the specificity of peptide showing high affinity for one or more alleles, modifications as described above are made and the resulting peptides are further tested for binding. Modified peptides which show significantly higher binding to particular MHC alleles are then selected. The modifications can be made in a more or less random manner. One approach to modifying the starting peptide is to combine binding motifs from two or more alleles.

An other approach may be used in which anchor residues that contain side chains critical for the binding to the MHC are inserted into a poly-alanine peptide of 13 residues. This approach has been used by Jardetzky et al., Nature 353, 326-329 (1990), who demonstrated that a polyalanine peptide which was modified with a single dominant MHC contact residue (Tyr) endowed the peptide with high affinity binding capacity for DR1. Instead of using tyrosine as the main MHC contact residue, cyclohexylalanine or phenylalanine can also be utilized. These residues are interchangeable with Tyr with respect to a peptide's capacity to bind those DR alleles capable of high affinity binding of the HA peptide, and furthermore also allow binding to MHC molecules that contained a G-V substitution at residue 86 in the DR  $\beta$  chain. This change affects the binding specificity of the B binding pocket in class II MHC such that tyrosine is no longer capable of binding effectively, whereas cyclohexylalanine, as well as phenylalanine, can bind.

The biological activity of the peptides identified above may be assayed in a variety of systems. Typically, the ability to inhibit antigen-specific T cell activation is tested. In one exemplary protocol, an excess of peptide is incubated with an antigen-presenting cell of known MHC expression, (e.g., DR1) and a T cell clone of known antigen specificity (e.g., tetanus toxin 830-843) and MHC restriction (again, DR1), and the antigenic peptide itself (i.e., tetanus toxin 830-843). The assay culture is incubated for a sufficient time for T cell proliferation, such as four days, and proliferation is then measured using standard procedures, such as pulsing with tritiated thymidine during the last 18 hours of incubation. The percent inhibition, compared to the controls which received no inhibitor, is then calculated.

The capacity of peptides to inhibit antigen presentation in an in vitro assay has been correlated to the capacity of the peptide to inhibit an immune response in vivo. In vivo activity may be determined in animal models, for example, by administering an antigen known to be restricted to the particular MHC molecule recognized by the peptide, and the



immunomodulatory peptide. T lymphocytes are subsequently removed from the animal and cultured with a dose range of antigen. Inhibition of stimulation is measured by conventional means, e.g., pulsing with [<sup>3</sup>H]-thymidine, and comparing to appropriate controls. Certain experimental details will of course be apparent to the skilled artisan. See also, Adorini, et al., *Nature* 334, 623-625 (1988), incorporated herein by reference.

A large number of cells with defined MHC molecules, particularly MHC Class II molecules, are known and readily available from, for instance, the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," 6th edition (1988) Rockville, Maryland, U.S.A., which is incorporated herein by reference.

A preferred embodiment of the peptides of the present invention comprises modifications to the N- and C-terminal residues. As will be well understood by the artisan, the N- and C-termini may be modified to alter physical or chemical properties of the peptide, such as, for example, to affect binding, stability, bioavailability, ease of linking, and the like.

Modifications of peptides with various amino acid mimetics or D-amino acids, for instance at the N- or C-termini, are useful for instance, in increasing the stability of the peptide in vivo. Such peptides may be synthesized as "inverso" or "retro-inverso" forms, that is, by replacing L-amino acids of a sequence with D-amino acids, or by reversing the sequence of the amino acids and replacing the L-amino acids with D-amino acids. As the D-peptides are substantially more resistant to peptidases, and therefore are more stable in serum and tissues compared to their L-peptide counterparts, the stability of D-peptides under physiological conditions may more than compensate for a difference in affinity compared to the corresponding L-peptide. Further, L-amino acid-containing peptides with or without substitutions can be capped with a D-amino acid to inhibit exopeptidase destruction of the antigenic peptide.

Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al., *Eur. J. Drug Metab. Pharmacokin.* 11, 291-302 (1986); Walter et al., *Proc. Soc. Exp. Biol. Med.* 148, 98-103 (1975); Witter et al., *Neuroendocrinology* 30, 377-381 (1980); Verhoef et al., *J. Endocrinology* 110, 557-562 (1986); Handa et al., *Eur. J. Pharmacol.* 70, 531-540 (1981); Bizzozero et al., *Eur. J. Biochem.* 122, 251-258 (1982); Chang, *Eur. J. Biochem.* 151, 217-224 (1985), all of which are incorporated herein by reference.

Stability may also be increased by introducing D-amino acid residues at the C and N termini of the peptide. Previous studies have indicated that the halflife of L-amino acid-containing peptides in vivo and in vitro, when incubated in serum-containing medium, can be extended considerably by rendering the peptides resistant to exopeptidase activity by introducing D-amino acids at the C and N termini.

The peptides or analogs of the invention can be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, e.g., those at critical contact sites, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins, such as L- $\alpha$ -amino acids, or their D-isomers, but may include non-protein amino acids as well, such as  $\beta$ - $\gamma$ - $\delta$ -amino acids, as well as many derivatives of L- $\alpha$ -amino acids. As discussed, a peptide of the present invention may generally comprise either L-amino acids or D-amino acids, but not D-amino acids within a core binding region.

#### CTL PEPTIDES

CTL peptides may be administered with the pan DR peptides of the invention to enhance an immune response. CTL epitopes from a number of antigenic proteins can be used in the conjugates of the present invention. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B

core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens.

5 In certain embodiments the CTL peptides of the invention are derived from within the HBV surface antigen or the nucleocapsid polypeptides, core and precore. In more preferred embodiments described herein the CTL-inducing peptides are derived from the region of HBenv<sub>309-328</sub> (peptide 10 799.08), HBenv<sub>329-348</sub> (peptide 799.09) HBenv<sub>349-368</sub> (peptide 799.10), or the region HBC<sub>91-110</sub> (peptide 802.03), where the numbering is according to Galibert et al., *Nature* 281, 646 (1979), which is incorporated herein by reference.

CTL peptides comprising the appropriate epitopes are 15 synthesized and then tested for their ability to bind to MHC Class I molecules in assays using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorimetry, peptide- 20 dependent class I assembly assays, and inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce 25 primary in vitro or in vivo CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as potential therapeutic agents.

The one or more CTL peptide may be administered with one or more pan DR peptide in a mixture which may or may not 30 involve noncovalent associations between the peptides. For instance, one or more of the peptides may be lipidated as described below to facilitate association between the peptides. Alternatively, the peptides may be covalently linked to form a CTL/pan DR conjugate.

35 To facilitate the association of the CTL peptide with the pan DR peptide, additional amino acids can be added to the termini of the CTL peptide. The additional residues can also be used for coupling to a carrier, support or larger

peptide, for reasons discussed herein, or for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH<sub>2</sub> acylation, e.g., by alkanoyl (C<sub>1</sub>-C<sub>20</sub>) or thioglycolyl acetylation, terminal-carboxy amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

As with the pan DR peptides, it will be understood that the peptides of the present invention or analogs thereof which have CTL stimulating activity may be modified to provide other desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide. For instance, the peptides can be modified by extending, decreasing or substituting in the peptides amino acid sequences by, e.g., the addition or deletion of amino acids on either the amino terminal or carboxy terminal end, or both, of peptides derived from the sequences disclosed herein. Usually, the portion of the sequence which is intended to substantially mimic a CTL stimulating epitope will not differ by more than about 20% from the sequence of the target antigenic protein, except where additional amino acids may be added at either terminus for the purpose of modifying the physical or chemical properties of the peptide for, e.g., ease of linking or coupling, and the like. In those situations where regions of the peptide sequences are found to be polymorphic among viral subtypes, it may be desirable to vary one or more particular amino acids to more effectively mimic differing cytotoxic T-lymphocyte epitopes of different viral strains or subtypes.

Within the peptide sequence regions identified by the present invention as containing CTL epitopes, e.g., HBV specific peptides, there are residues (or those which are

substantially functionally equivalent) which allow the peptide to retain their biological activity, i.e., the ability to stimulate a class I-restricted cytotoxic T-lymphocytic response against virally infected cells or cells which express viral antigens. These residues can be identified by single amino acid substitutions, deletions, or insertions. In addition, the contributions made by the side chains of the residues can be probed via a systematic scan with a specified amino acid (e.g., Ala). Peptides which tolerate multiple substitutions generally incorporate such substitutions as small, relatively neutral molecules, e.g., Ala, Gly, Pro, or similar residues. The number and types of residues which can be substituted, added or subtracted will depend on the spacing necessary between the essential epitopic points and certain conformational and functional attributes which are sought (e.g., hydrophobicity vs. hydrophilicity). If desired, increased binding affinity of peptide analogues to its MHC molecule for presentation to a CTL can also be achieved by such alterations. Generally, any spacer substitutions, additions or deletions between epitopic and/or conformationally important residues should employ amino acids or other moieties chosen to avoid steric and charge interference which might disrupt binding. Peptides which tolerate substitutions while retaining the desired biological activity may also be synthesized as D-amino acid containing peptides, as described above for Pan DR peptides.

The peptides of the invention can be combined via linkage to form polymers (multimers), or can be formulated in a composition without linkage, as an admixture. Where the same peptide is linked to itself, thereby forming a homopolymer, a plurality of repeating epitopic units are presented. When the peptides differ, e.g., a cocktail representing different viral subtypes, different epitopes within a subtype, different HLA restriction specificities, or peptides which contain T helper epitopes, heteropolymers with repeating units are provided. In addition to covalent linkages, noncovalent linkages capable of forming

intermolecular and intrastructural bonds are also contemplated.

#### PREPARATION OF THE CONJUGATES

5 As noted above, CTL inducing peptides may be covalently linked to the pan DR-binding peptides to prepare conjugates of the invention. Particularly preferred CTL inducing peptides/pan DR-binding conjugates are linked by a spacer molecule. Alternatively, the CTL peptide may be linked to the pan DR-binding peptide without a spacer. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions and may have linear or branched side chains. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. In certain preferred embodiments herein the neutral spacer is Ala. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. Preferred exemplary spacers are homo-oligomers of Ala. When present, the spacer will usually be at least one or two residues, more usually three to six residues. In other embodiments the pan DR-binding peptide is conjugated to the CTL peptide, preferably with the pan DR-binding peptide positioned at the amino terminus. The peptides may be joined by a neutral linker, such as Ala-Ala-Ala or the like, and preferably further contains a lipid residue such as palmitic acid or the like (as described further below) which is attached to alpha and epsilon amino groups of a Lys residue ((PAM)<sub>2</sub>Lys), which is attached to the amino terminus of the peptide conjugate, typically via Ser-Ser linkage or the like.

35 The CTL inducing peptide may be linked to the pan DR-binding peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the CTL inducing peptide or the pan DR-binding peptide may be acylated. In addition, the CTL peptide/pan DR-binding conjugate may be linked to certain

alkanoyl ( $C_1$ - $C_{20}$ ) lipids via one or more linking residues such as Gly, Gly-Gly, Ser, Ser-Ser as described below.

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which assists in priming CTL. Lipids have been identified as agents capable of assisting the priming CTL in vivo against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine ( $P_3$ CSS) can be used to prime virus specific CTL when covalently attached to an appropriate peptide. See, Deres et al., *Nature* 342, 561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to  $P_3$ CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with  $P_3$ CSS conjugated to a peptide which displays an appropriate epitope, the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

#### PHARMACEUTICAL COMPOSITIONS

The pan DR-binding peptides of the present invention and pharmaceutical and vaccine compositions thereof can be administered to mammals, particularly humans, for prophylactic and/or therapeutic purposes. The pan DR peptides can be used

to enhance immune responses against other immunogens administered with the peptides. For instance, CTL/pan DR mixtures may be used to treat and/or prevent viral infection and cancer. Alternatively, immunogens which induce antibody responses can be used. Examples of diseases which can be treated using the immunogenic mixtures of pan DR peptides and other immunogens include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV and condyloma acuminatum.

The pan DR-binding peptides may also be used to treat a variety of conditions involving unwanted T cell reactivity. Examples of diseases which can be treated using pan DR-binding peptides include autoimmune diseases (e.g., rheumatoid arthritis, multiple sclerosis, and myasthenia gravis), allograft rejection, allergies (e.g., pollen allergies), Lyme disease, hepatitis, LCMV, post-streptococcal endocarditis, or glomerulonephritis, and food hypersensitivities.

In therapeutic applications, the immunogenic compositions or the pan DR-binding peptides of the invention are administered to an individual already suffering from cancer, autoimmune disease, or infected with the virus of interest. Those in the incubation phase or the acute phase of the disease may be treated with the pan DR-binding peptides or immunogenic conjugates separately or in conjunction with other treatments, as appropriate.

In therapeutic applications, compositions comprising immunogenic compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to cure or at least partially arrest symptoms and/or complications. Similarly, compositions comprising pan DR-binding peptides are administered in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being



treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

Therapeutically effective amounts of the immunogenic compositions of the present invention generally range for the  
5 initial immunization (that is for therapeutic or prophylactic administration) from about 1.0  $\mu$ g to about 5000  $\mu$ g of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0  $\mu$ g to about 1000  $\mu$ g of peptide pursuant to a  
10 boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood.

Therapeutically effective amounts of the DR-binding peptides of the present invention generally range from about  
15 0.1 mg to about 2,000 mg of peptide per day for a 70 kg patient, with dosages of from about 0.5 mg to about 1,000 mg of peptide per day being more commonly used.

It must be kept in mind that the compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life  
20 threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the conjugates, it is possible and may be felt desirable by the treating physician to administer  
substantial excesses of these compositions.

For prophylactic use, administration should begin at  
25 the first sign of disease or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter.  
30 In chronic infection, loading doses followed by boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those  
35 individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or

during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide mixtures or conjugates can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers. It is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic infection, a representative dose is in the range of about 1.0  $\mu\text{g}$  to about 5000  $\mu\text{g}$ , preferably about 5  $\mu\text{g}$  to 1000  $\mu\text{g}$  for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the peptides or conjugates dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting

and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

5           The concentration of pan DR and/or CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, 10 viscosities, etc., in accordance with the particular mode of administration selected.

          The peptides and conjugates of the invention may also be administered via liposomes, which serve to target the conjugates to a particular tissue, such as lymphoid tissue, or 15 targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is 20 incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide 25 or conjugate of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged 30 phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., 35 Szoka et al., *Ann. Rev. Biophys. Bioeng.* 9, 467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide or conjugate may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the conjugate being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more conjugates of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of conjugates are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an

immunogenically effective amount of an immunogenic pan DR peptide or a CTL\pan DR peptide conjugate as described herein. The conjugate(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the virus or tumor cells. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as bovine serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P<sub>3</sub>CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

Vaccine compositions containing the pan DR peptides of the invention are administered to a patient susceptible to or otherwise at risk of disease, such as viral infection or cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0  $\mu$ g to about 5000  $\mu$ g per 70

kilogram patient, more commonly from about 10  $\mu$ g to about 500  $\mu$ g per 70 kg of body weight.

5 In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens.

10 For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby  
15 elicits a host CTL response. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al., *Nature* 351, 456-460 (1991)  
20 which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., *Salmonella typhi* vectors and the like, will be apparent to those skilled in the art from the description herein.

25 Antigenic conjugates may be used to elicit CTL *ex vivo*, as well. The resulting CTL, can be used to treat chronic infections (viral or bacterial) or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a peptide vaccine approach of therapy. *Ex vivo* CTL responses to a particular pathogen (infectious agent  
30 or tumor antigen) are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-4 weeks), in which the CTLp are activated and  
35 mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell).

The peptides of this invention may also be used to make monoclonal antibodies. Such antibodies may be useful as potential diagnostic or therapeutic agents.

The peptides may also find use as diagnostic reagents. For example, a peptide of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the peptides may also be used to predict which individuals will be at substantial risk for developing chronic infection.

The following examples are offered by way of illustration and not by way of limitation.

#### EXAMPLES

##### **EXAMPLE I**

##### **Experimental Procedures**

##### **I. Cell Lines and MHC Purification**

Various cell lines were used as sources of purified human and mouse class II molecules. The following Epstein-Barr virus (EBV) transformed homozygous cell lines were used as sources of human HLA class II molecules (Valli et al., J. Clin. Invest. 91, 616-628, 1993): LG2 [DRB1\*0101 (DR1)]; 3107 [DRB1\*1501 (DR2w2b)]; MAT [DRB1\*0301 (DR3)]; PREISS [DRB1\*0401 (DR4)]; BIN40 [DRB1\*0404 (DRw14)]; SWEIG [DRB1\*11011 (DR5)]; PITOUT [DRB1\*0701 (DR7)]; PF [DQA1\*0301/DQB1\*0301 (DQ3.1)]. In some instances, transfected fibroblasts were used: L416.3 [DRB5\*0101 (DR2w2a)]; TR81.19 [DRB3\*0101 (DR52a)]; and L257.6 [DRB4\*0101 (DRw53)]. For mouse class II molecules, the following cell lines were used: A20 (IA<sup>d</sup>, IE<sup>d</sup>) (Sette et al., Science 258, 1801-1804, 1992); CH12 (IA<sup>k</sup>, IE<sup>k</sup>) (Sette et al., 1992); LS102.9 (IA<sup>s</sup>) (Wall et al., Int. Imm. 4, 773-777, 1992); and DB27.4 (IA<sup>b</sup>) (Wall et al., J. Immuno. 152:4526-4536, 1994).

## II. Purification of MHC molecules

MHC molecules were purified essentially as described (Gorga et al., *J. Biol. Chem.* 262, 16087-16094 (1987)). Briefly, human class II molecules were purified by affinity chromatography using the LB3.1 (all DR, Valli et al., *J. Clin. Invest.* 91, 616-628 (1993)) or the IVD12 (DQ3.1, Sidney et al., *J. Immunol.* 152, 4516-4525 (1994)) monoclonal antibodies. Mouse class II molecules were purified by the use of the MKD6 (IA<sup>d</sup>, Sette et al., *Science* 258, 1801-1804 (1992)); 10.3.6 (IA<sup>k</sup>, Sette et al., *supra*); 14.44 (IE<sup>d</sup> and IE<sup>k</sup>, Sette et al., *supra*); and Y3JP (IA<sup>s</sup>, Wall et al., *Int. Immunol.* 4, 773-777 (1992)) monoclonal antibodies.

## III. Peptide Synthesis

Peptides were synthesized by sequential coupling of N- $\alpha$ -Fmoc-protected amino acids on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer using standard Fmoc coupling cycles (software version 1.40). All amino acids, reagents, and resins were obtained from Applied Biosystems or Nova Biochem (San Diego, CA). Solvents were obtained from Burdick & Jackson. Solid-phase synthesis was started from an appropriately substituted Fmoc-amino acid-Wang resin. The loading of the starting resin was 0.5-0.7 mmol/g polystyrene, and 0.1 or 0.25 meq were used in each synthesis. A typical reaction cycle proceeded as follows: The N-terminal Fmoc group was removed with 25% piperidine in dimethylformamide (DMF) for 5 min, followed by another treatment with 25% in DMF for 15 min. The resin was washed 5 times with DMF. An N-methylpyrrolidone (NMP) solution of a 4 to 10-fold excess of a preformed 1-hydroxybenzotriazole ester of the appropriate Fmoc-amino acid was added to the resin, and the mixture was allowed to react for 30-90 min. The resin was washed with DMF in preparation for the next elongation cycle. The fully protected, resin-bound peptide was subjected to a piperidine cycle to remove the terminal Fmoc group. The product was washed with dichloromethane and dried. The resin was then treated with trifluoroacetic acid in the presence of appropriate scavengers [e.g., 5% (v/v) in water] for 60 min at



20°C. After evaporation of excess trifluoroacetic acid, the crude peptide was washed with diethylether, dissolved in water, and lyophilized. The peptides were purified to >95% homogeneity by reverse-phase HPLC using H<sub>2</sub>O/CH<sub>3</sub>CN gradients containing 0.8% TFA modifier on a Vydac, 300A pore-size, C-18 preparative column. The purity of the synthetic peptides was assayed on an analytical reverse-phase column and their composition ascertained by amino acid analysis and/or sequencing. The cyclohexylalanine used in the synthetic procedures was purchased from Nova Biochem (San Diego, CA). Palmitylated peptides were produced by coupling palmitic acid on the resin before cleaving the peptide. Coupling was accomplished by a symmetrical anhydride method, i.e., twofold excess of palmitic acid and one-fold of diisopropylcarbodiimide in dichloromethane for 1 hr.

#### IV. MHC Peptide Binding Assays

Purified mouse or human class II molecules (5 to 500 nM) were incubated with 5 nM <sup>125</sup>I-radiolabeled peptides for 48 hr in PBS containing 5% DMSO in the presence of a protease inhibitor mixture. Purified peptides were iodinated using the chloramine-T method (Buus, et al., *Science* 235, 1353-1358 (1987)). The final concentrations of protease inhibitors were: 1 nM PMSF, 1.3 mM 1.10 phenanthroline, 73 μM pepstatin A, 8mM EDTA, 6 mM N-ethylmaleimide, and 200 μM Nα-p-tosyl-L-lysine chloromethyl ketone. Final detergent concentration in the incubation mixture was 2.6% digitonin (IA<sup>d</sup> and IA<sup>k</sup>) or 0.05% NP-40 (all other class II molecules). Class II-peptide complexes were separated from free peptide by gel filtration on Sephadex G-50 or TSK2000 columns, and the fraction of peptide bound was calculated as previously described (Sette et al., *J. Immunol.* 142, 35-40 (1989)). In preliminary experiments, each of the DR preparations was titrated in the presence of fixed amounts of radiolabeled peptides to determine the concentration of class II molecules necessary to bind 10 to 20% of the total radioactivity. All subsequent inhibition and direct binding assays were then performed using this class II concentration. In the

inhibition assays, inhibitory peptides were typically tested at concentrations ranging from 120  $\mu\text{g/ml}$  to 1.2  $\text{ng/ml}$ . The data were then plotted, and the dose yielding 50% inhibition was measured. Each peptide was tested in two to four completely independent experiments.

As used herein binding at <50 nM constitutes high affinity and binding at 50-500 nM constitutes intermediate affinity binding.

#### V. Inhibition of DR Restricted Peptide Presentation

The capacity of peptides to block the antigen presenting function of MHC was assayed by incubating mitomycin C-treated EBV cells of the appropriate DR type ( $5 \times 10^4/\text{well}$ ) with inhibitor peptides in RPMI 1640 (Bio Whittaker, Walkersville, MD) in complete medium containing 10% human serum (Gemini Bioproducts, Inc., Calabasas, CA). The inhibitor peptides were routinely titrated in 96-well U-bottom plates (Costar, Cambridge, MA) over a range of four ten-fold dilutions starting at a concentration of 150  $\mu\text{g/ml}$ . Along with inhibitor peptide, assay wells also received suboptimal concentrations of the HA 307-319 peptide (DR1, DR4w4, DR5, DR52b) or HA 307-319, Y<sub>309</sub>>F (DR4w14), or Lol P1 171-190 (DR3) (Sidney et al., *J. Immunol.* 149, 2634-2640 (1992)), which, in the absence of inhibitor peptides, resulted in 30-50% of the maximal proliferative response. This concentration was routinely 50 to 200  $\text{ng/ml}$ . After incubating APC with peptides for 2 hr at 37°C in a 5% CO<sub>2</sub> incubator,  $2 \times 10^4$  T cells were added to each well. The T cell clones used were Cl 1 [DR1 and DR52b (Krieger et al., *J. Immunol.* 146, 2331-2340 (1991))]; Clone 42.19 (DRw14); Clone JK1 (DR5); and line 132-132 (DR3). The proliferation of the T cells was measured three days later. Briefly, 24 hr after T cell addition, [<sup>3</sup>H]thymidine (1  $\mu\text{Ci/well}$ ) (ICN, Irvine, CA) was added to each well for a final 18 hr incubation. Cells were then harvested onto glass fiber filters (LKB Wallac cell harvester 1295-001, LKB, Gaithersburg, MD), and thymidine incorporation (LKB betaplate counter 1205) was measured. The percent inhibition of antigen

presentation was calculated for each dose of inhibitor peptide required to inhibit 50% of the proliferative response.

5

**EXAMPLE 2****DR Binding Specificity of "Universal" Peptide Epitopes**

10 The binding motifs of several murine and human class II MHC alleles have been defined, and motif analysis by sequencing of naturally processed peptides has also recently been described for various class II types (Rudensky et al., Nature 353, 622-627 (1991); Chicz et al., Nature 358, 764-768 (1992); Hunt et al., Science 256, 1817-1820 (1992); Rudensky et al., Nature 359, 429-431 (1992)).

15 In the case of DR molecules in particular, it has been shown (Brown et al., Nature 364, 33-39 (1993)) that a large hydrophobic anchor engaging a corresponding hydrophobic pocket of the MHC binding groove is the most crucial determinant of peptide-DR interactions. Several other anchors play definite, albeit less prominent roles and help determine allelic specificity. Recently it has also been emphasized that the peptide backbone of the C-terminal half of the peptide molecule is engaged in direct hydrogen bonding with the walls of the MHC binding groove.

20 Although the allele-specific polymorphic residues that line the peptide binding pockets of MHC tend to endow each allele with the capacity to bind a unique set of peptides, there are many instances in which a given peptide has been shown to bind to more than one MHC specificity. This has been best documented in the case of the human DR isotype, in which it had been previously noted that several DR alleles appeared to recognize similar motifs, and independently, several investigators reported degenerate binding and/or recognition of certain epitopes in the context of multiple DR types, leading to the concept that certain peptides might represent "universal" epitopes (Busch et al., Int. Immunol. 2, 443-451 (1990); Panina-Bordignon et al., Eur. J. Immunol. 19, 2237-2242 (1989); Sinigaglia et al., Nature 336, 778-780

(1988); O'Sullivan et al., *J. Immunol.* 147, 2663-2669 (1991); Roache et al., *J. Immunol.* 144, 1849-1856 (1991); Hill et al., *J. Immunol.* 147, 189-197 (1991)).

The DR binding capacity of previously described DR binding peptides capable of binding more than one DR molecule (HA 307-319, TT 830-843, CS 378-398, MT 17-31, and HBVnc 50-69) was established using the assay described in Example I, paragraph V. The data obtained (Table II, section A) demonstrate that although these peptides were indeed capable of binding several of the DR molecules tested, they failed to bind to others. For example, the HA 307-319 bound with high (<50 nM) or intermediate (50-500 nM) affinity to DR1, DR4w4, DR5, DR7, and DR2w2a, and weakly to DRw53 (2.2  $\mu$ M); while no binding was detectable for the remaining four DR specificities. HBVnc 50-69 also bound 5 of the 10 DR specificities tested (DR1, DR2w2b, DR4w4, DR5, and DR2w2a) with high or intermediate affinity. TT 830-843 and CS 378-398 bound with high or intermediate affinity to 4/10 DR molecules tested (DR1, DR5, DR7, DR2w2a and DR1, DR4w4, DR5, DR7, respectively) and MT 17-31 bound with high or intermediate affinity to 3/10 of the DR types.

In conclusion, although these previously described "universal" epitopes bound to several DR types, they were not completely cross-reactive in their binding capacity, in that a maximum of 50% of the DR specificities tested bound a given peptide with high to intermediate affinity.

### EXAMPLE 3

#### Development of Peptides with High Affinity for Multiple DR Alleles: 760.50 and 760.57

A number of peptides capable of binding with high affinity to the rheumatoid arthritis associated DR alleles DR1, DR4w4, and DR4w14 were generated. To produce these peptides, we used a strategy initially described by Jardetzky et al., *EMBO J.* 9:1797-1803 (1990), in which anchor residues that contain side chains critical for the binding to MHC are inserted into a poly-alanine peptide of 13 residues. Two such

peptides, designated 760.50 and 760.57, which are described in  
copending parent application 08/121,101 and which were  
particularly interesting with regard to their broad DR binding  
specificity. When tested for binding to a panel of 10  
5 purified DR molecules, it was found that, in general, these  
peptides bound with higher affinity and broader specificity  
than the natural "universal" epitopes described above (Table  
II, section B). Neither 760.50 nor 760.57 was completely  
cross-reactive, since only low affinity binding was detected  
10 in 4 of the 10 alleles analyzed (DR2w2b, DR3, DR52a, and  
DRw53).

Table II: Binding Capacity of Various Peptide Epitopes to Different DR Alleles  
TABLE II

TABLE II

Peptide	Sequence	DRβ1 Alleles				DRβ2 Alleles					
		DR1	DR2w2b	DR3	DR4w4	DR4w1 4	DR5	DR7	DR52 a	DRw53	DR2w 2a
A	HA 307-319	5 (1)	-- (2)	--	45	--	118	385	--	2200	45
	PKYVKONTLKLAT	70	9.1	--	85	505	263	676	2765	ND (4)	211
	PHHTALROAILCWGEL	52	--	3623	--	--	20	25	--	--	20
	HBVnc 50-69	17	1820	--	250	2272	154	147	--	--	1430
	MTLA	13	--	--	--	--	--	208	6266	6538	350
B	TT 830-843										
	DIFKKIAKMFKARRVFN										
	VVNR										
C	CS 378-398										
	YSGPLKAEIAQRLEDV										
D	MT (Y)17-31										
		3.1	569	6410	2.8	6.9	6.1	192	9400	560	57
E	760.50	4.5	479	2550	2	3.1	5.4	78	--	3300	5
	aA(X)IAAAATLKAAa										
F	906.09	0.61	14	280	2.6	5.4	2.5	76	588	93	2.0
	aA(X)IAAAATLKAAa	0.38	19	100	2.8	3.3	2.4	31	1120	41	1.3
G	906.11										
	aA(X)IAAAATLKAAa										
H	965.10	0.91	40	86	1.1	9.1	9.1	167	979	75	6
	aK(X)IAAAATLKAAa	1.2	27	1470	2	8	18	208	797	420	11
I	1024.03										
	aKFVAAATLKAAa										

(1) nM IC50% values

(2) dashes indicate no detectable binding (>10,000 nM)

(3) X = cyclohexalanine

(4) ND = not done

(1) nM IC50% values

(2) dashes indicate no detectable binding (&gt;10,000 nM)

(3) X = cyclohexylalanine

(4) ND = not done

**EXAMPLE 4****Development of Peptides with High Affinity for Multiple DR Alleles: 906.09 and 906.11**

5           To further broaden specificity the 906.09 and 906.11 peptides were synthesized, in which a V or I was introduced at position 4 of the 760.57 peptide. As shown in Table II, section C, both 906.09 and 906.11 peptides retained the good binding affinity for DR1, DR2w2a, DR4, DR5, and DR7 (in the  
10           range of 0.3 to 80 nM). Furthermore, the binding capacity (in comparison to 760.50 and 760.57) for molecules DR2w2b, DR3, DR52a, and DRw53 was significantly improved (10- to 25-fold), with IC50% in the range of 20 to 1200 nM. Thus, 9 of 10 DR specificities bound tested bound these peptides with high or  
15           intermediate affinity, and one, DR52a, bound weakly.

          In conclusion, these data illustrate the development of peptides binding with high affinity to most, if not all, DR alleles. Because of this broad cross-reactivity pattern amongst different DR molecules, we have determined that the  
20           906.09 and 906.11 peptides are Pan DR binding peptides.

**Example 5****Pan DR Binding Peptides Also Bind DQ3.1 and Mouse Class II Molecules**

25           Assays were carried out to determine whether the Pan DR binding peptides were also capable of binding other human class II isotypes or non-human class II molecules. More specifically, the binding capacity of the Pan DR binding  
30           peptides to DQ3.1 and several mouse class II specificities was determined, as shown in Table III. For reference purposes, the binding affinities of previously described mouse class II epitopes are also shown in Table III, section A. All of these previously described epitopes bound their relevant restriction  
35           elements with high or intermediate affinity, between 20 and 400 nM. It was found that, in general, the 760 series peptides (Table III, section B) bound with intermediate affinity, in the range of 80 to 700 nM, to five of the six

alleles tested (IA<sup>b</sup>, IA<sup>d</sup>, IE<sup>d</sup>, IA<sup>s</sup>, IE<sup>k</sup>). Interestingly, the 906 series peptides (Table III, section C) bound with significantly higher affinity, in the 10 to 100 nM range in the case of the alleles mentioned above, and 906.11 also bound with intermediate affinity to IA<sup>k</sup>. With respect to binding to DQ3.1, it was found that 760.50, 760.57, 906.09, and 906.11 all bound with relatively high affinity to purified DQ3.1 molecules (in the 30 to 120 nM range).

As a control, the binding potential of the 760 and 906 peptides to human class I molecules was also examined. No binding was detected, up to 10  $\mu$ M, to purified HLA-A1, -A2.1, -A3, -A11, and -A24 molecules (data not shown). In conclusion, these data suggest that the 906 series peptides are Pan class II (but not class I) MHC binding peptides.



Table III: Capacity of Various Peptide Epitopes to Bind Purified DQ 3.1 and Mouse Class II Molecules  
TABLE III

Peptide/Restriction element(s)	Sequence	Class II Alleles					
		DQ3.1	IA b	IA d	IE d	IA s	IE k
A	HBVc 128-140/IA b	ND (1)	255	--	--	--	--
	Ova 323-336/IA d, b	577 (2)	400	110	--	1038	1000
	Lambda rep. 12-26/IE	-- (3)	--	1100	170	--	--
	d, k	--	>3100	--	--	86	--
	PLP 139-151/IA s	3750	7000	1222	8500	--	20
B	HEL 46-61/IA k						
	760.50	31	200	688	155	491	10,00
	760.57	94	377	192	172	120	0
							5260
C	906.09	48	31	38	31	104	1333
	906.11	115	28	25	13	98	154
D	965.10	25	94	733	354	613	3333
	1024.03	23	44	1133	3056	1059	--

(1) ND = not done

(2) nM IC50% values

(3) dashes indicate no detectable binding (&gt;10,000 nM)

## EXAMPLE 6

## Inhibition of T Cell Proliferation by Pan DR Binders

5           Because of their degenerate class II binding capacity, the Pan DR binders are candidates as therapeutics in the inhibition of T cell mediated events involved in allograft rejection, allergic responses, or autoimmunity. Accordingly, the capacity of these peptides to block an antigen-specific in vitro T cell proliferative response was evaluated. The inhibition of antigen presentation assay described in Example 1, paragraph (V) was used to make these evaluations.

10           In keeping with their MHC binding capacity, it was found that these peptides were potent inhibitors of the proliferative responses of human T cells restricted by at least six different DR molecules (Table IV). More specifically, peptides 760.50 and 760.57, which have high binding affinities for DR1, DR4w4, DR4w14, and DR5, inhibited T cell proliferation restricted by those alleles, with IC50% in the 1.0 to 25  $\mu$ M range. By contrast, these peptides bound DR3 molecules only weakly, in the 2.5 to 6.5  $\mu$ M range, and accordingly, DR3 restricted T cell activation was inhibited poorly (IC50% of 220  $\mu$ M for 760.57) or not at all (IC50% of >250  $\mu$ M for 760.50).

25           The 906.09 and 906.11 peptides also inhibited DR1, DR4w4, DR4w14, and DR5 responses quite effectively (IC50% in the 0.5 to 15  $\mu$ M range). As expected, the 906 analogs, which have intermediate DR3 binding capacity, were also capable of inhibiting DR3 restricted antigen presentation, with IC50% in the 30 to 60  $\mu$ M range.

30           In the same set of experiments, we also tested the 760 and 906 peptides for their capacity to inhibit a DR52b restricted response. This experiment was of interest to us inasmuch as we have not yet been able to develop a molecular binding assay to measure peptide binding to DR52b molecules. The data obtained demonstrate that both 906.09 and 906.11 peptides inhibited the presentation of HA 307-319 Clone 1 in

the context of DR52b molecules with good IC50%, in the 1 to 2  $\mu$ M range, thereby extending to an eleventh allele the Pan DR binding capacity of these peptides.

Finally, these peptides failed to inhibit  
5 proliferation of the HA-specific, DR-restricted T cell clone  
in response to the polyclonal mitogen PHA, and also failed to  
inhibit in the recently described T cell antagonist assay (De  
Magistris et al., Cell 68, 525-634 (1992)), in which peptides  
are added subsequent to (not simultaneously with) the  
10 antigenic stimulus (data not shown). These findings rule out  
the possibility that the results described above might have  
been caused by some non-specific cytotoxicity of the 760 or  
906 peptides.

TABLE IV: Inhibition of T Cell Proliferation by Pan DR Binding Peptides

PEPTIDE	SEQUENCE	Activity in inhibition of antigen presentation assay restricted by:						
		DR1	DR3	DR4w4	DR4w14	DR5	DR52b	
760.50	aAIXIAAAKTAAAAa	4.3 (1)	>250	3.2	2.8	25	>180	
760.57	aAIXIAAAKTLKAAa	2.1	220	0.94	0.79	18	7.5	
906.09	aAIXIVAAATLKAAa	0.88	31	0.58	0.43	11	1.6	
906.11	aAIXIAAATLKAAa	1	56	0.74	0.59	13	1.7	

(1)  $\mu$ M IC50%

**EXAMPLE 7****Generation of Pan DR T Cell Epitopes by Modification of  
Broadly Reactive Class II Binding Peptides**

5

A different type of application of Pan DR binding peptides was also considered, namely to use these peptides to produce Pan DR restricted T helper epitopes that could provide help for both humoral and cytotoxic responses. Because all of the potential TCR contact residues in the Pan DR binding peptides were alanines, it was hypothesized that due to the limited interactions the methyl side chains could participate in, introduction of more bulky hydrophobic charged residues might improve the likelihood of interactions with T cell receptors and thereby increase their immunogenicity.

Following this line of reasoning, we further modified the 906.09 Pan DR peptide. Several analogs were generated by introducing bulky or charged side groups at positions 2, 5, and 7, which are potential TCR contact residues based on previous analysis of the HA 307-319 peptide. By contrast, those positions known to influence DR binding were left undisturbed (3, 4, 8, 9, and 11). In addition, analogs were also generated that carried the natural amino acid, Phe, instead of cyclohexylalanine at position 3. These peptides were then tested for retention of their capacity to bind multiple DR alleles, and those peptides that had no significant decrease in their DR binding capacity were then tested for their capacity to induce an immune response. The data from two of the best peptides, 965.10 and 1024.03, are discussed below.

When these two peptides were tested for HLA DR and murine Ia binding (Table II, section D and Table III, section D), it was found that, in general, they retained the high binding capacity and broad reactivity associated with the parent peptide, 906.09, for most DR alleles. The exceptions to this were represented by 1024.03 binding only weakly to DR3 (1470 nM) and also binding with intermediate (420 nM) rather than high affinity to DRW53. Also, the 965.10 and 1024.03

peptides showed greatly reduced binding capacities for most of the murine class II molecules tested. Good binding capacity was retained, however, for the 1A<sup>b</sup> allele, thus allowing H-2<sup>b</sup> mice to be used to test the in vivo immunogenicity of these peptides (see below). Finally, good DQ3.1 binding capacity of both peptides (in the 25 nM range) was also retained.

#### EXAMPLE 8

##### In Vitro Immunogenicity of Pan DR Binding Peptides

The Pan DR epitope 965.10, along with two of its progenitor peptides, 906.09 and 760.50 and the previously described natural epitope, TT 830-843, were compared for their capacity to stimulate in vitro T cell responses in PBMC from normal individuals. The protocol used entailed repeated stimulation of PBL with autologous APC and peptide antigens, and was specifically designed to allow the study of primary in vitro responses. This protocol is provided below, followed by the results of the assays.

20

##### A. Assay Protocol

PBMCs from healthy donors were stimulated in vitro using a protocol adapted from Manca et al., *J. Immunol.* 146, 1964-1971 (1991). Peripheral blood mononuclear cells (PBMC) were purified over Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) and plated in 4 wells of a 24-well tissue culture plate (Costar, Cambridge, MA) at  $4 \times 10^6$  PBMC/well. The peptides were added at a final concentration of 10  $\mu$ g/ml. Cultures were then incubated at 37°C, 5% CO<sub>2</sub>. On day 4, recombinant IL-2 was added at a final concentration of 10 ng/ml. Cultures were routinely fed every three days thereafter by aspirating off 1 ml of media and replacing it with fresh medium containing IL-2. Two additional stimulations of the T cells with antigen were performed on approximately days 14 and 28. The T cells ( $3 \times 10^5$ /well) were stimulated with peptide (10  $\mu$ g/ml) using autologous PBMC cells [ $2 \times 10^6$  irradiated (7500 rad)/well] as antigen presenting cells in a total of 3 wells of a 24-well tissue culture plate.

In addition, on days 14 and 28, T cell proliferative responses were determined as follows:  $2 \times 10^4$  T cells/well;  $1 \times 10^5$  irradiated PBMC/well as APC; the peptide concentration was titrated between 0.01-10  $\mu$ g/ml final concentration in U-bottom 96 well tissue culture plates (Costar, Cambridge, MA). The T cell proliferation assays were harvested on day 3 as described above.

#### B. Results

Representative data from three normal donors are shown in Figure 1. The data obtained following two rounds of stimulation are shown in panels A to C, and after a third round of stimulation, in panels D to F. As predicted, the parental peptides 760.50 and 906.09 were poorly immunogenic in these experiments. Neither peptide induced a significant ( $>10,000$  cpm) response following two rounds of stimulation. After a third round of stimulation, 760.50 induced a response in one donor of the three tested. The natural "universal" epitope TT 830-843 also failed to give a significant response after two rounds of stimulation, and after the third round of stimulation, TT 830-843 also generated a modest positive response in all three donors. In contrast to these weak responses, all three donors responded briskly after only two rounds of stimulation to the modified Pan DR peptide 965.10.

Figures 2A and 2B summarize all the in vitro stimulation data that have been obtained (second and third stimulations, respectively). After two in vitro stimulations (Fig. 2A), peptide 965.10 was the only peptide able to significantly stimulate T cells in the majority of donors (9/12). TT 830-843 was able to generate a response in fewer individuals (3/12), while 760.50 and 906.09 both failed to stimulate any response (0/3). By the third stimulation (Figure 2b) 965.10 generated significant responses in 11 of 12 donors tested, the TT 830-843 was now able to mount a significant response in a majority of the donors (7/12); 760.50 induced a response in 1 of 3 donors, and 906.09 failed to stimulate any of the 3 donors tested.

The ability of 965.10 to expand specific T cell populations in vitro as early as the second stimulation, plus its ability to give a significant T cell response in virtually all of the donors by the third stimulation, demonstrates the superior immunogenic capacity of this peptide relative to peptides TT 830-843, 760.50 or 906.09.

#### EXAMPLE 9

##### In Vivo Immunogenicity of the Pan DR Epitopes in Mice

The in vivo immunogenicity of the 760.50, 965.10, and 1024.03 peptides was tested in C57BL/6J (H-2<sup>b</sup>+) mice.

To carry out these assays, C57BL/6J mice were injected subcutaneously at the base of the tail with a dose titration of various peptides (0.000125, 0.0025, 0.05, 1, and 20  $\mu$ g/mouse) in PBS/CFA (Difco, Detroit, MI) in a 100  $\mu$ l volume. On day 10, inguinal and paraaortic lymph nodes from groups of three mice/peptide dose were collected, pooled, and homogenized into single cell suspensions. Cells were washed two times and subsequently plated ( $1 \times 10^6$  cells/well) in 96-well microtiter tissue culture plates. A log dose peptide titration (0.01 to 100  $\mu$ g/ml) of the immunizing peptide was added and a standard three day T cell proliferation assay performed as described above.

In these experiments, the activity of the non-natural epitopes to two other previously defined natural IA<sup>b</sup> restricted epitopes, Ova 323-336 and HBV core 128-140, were compared. These two natural epitopes bound with a somewhat lower (3 to 14 fold) affinity than 965.10 (Table III, section A). The TT 830-843 peptide, which did not bind IA<sup>b</sup> appreciably (data not shown), was included as a negative control. Groups of mice were immunized with varying amounts of peptide (0.000125 to 20  $\mu$ g/mouse) in CFA. Ten days following immunization, the draining lymph nodes were collected and stimulated in vitro with varying doses of antigen.



As shown in Figure 3, it was found that, consistent with its inability to bind IA<sup>b</sup>, TT 830-843 was unable to generate a specific T cell proliferative response. The known IA<sup>b</sup> restricted helper epitopes Ova 323-336 (Figure 3, panel B) and HBVc 125-140 (Figure 3, panel C) induced responses in the 25,000 to 70,000 cpm range at the two highest peptide doses used for immunization (1 and 20 µg/mouse). The Pan DR epitopes 965.10 (Figure 3, panel D) and 1024.03 (Figure 3, panel E) stimulated the strongest responses, with effective immunizing doses being obtained with as little as 0.05 µg/mouse, with a magnitude in the 100,000 to 150,000 cpm range. In contrast, peptide 760.50 (Figure 3, panel F) was only marginally immunogenic, with a very weak proliferative response being induced, and only at the highest (20 µg/mouse) dose tested. These results indicate that the Pan DR epitopes 965.10 and 1024.03 function as highly effective helper epitopes in vivo, as well as in vitro. Together with the human immunogenicity data, they also suggest that in addition to high MHC binding capacity, the presence of "immunodominant" amino acid residues at potential TCR contact positions is an important element for the generation of vigorous T cell responses.

#### EXAMPLE 10

##### Pan DR Peptides Act as Helper Epitopes For In Vivo CTL Induction in Mice

It is generally assumed that the capacity to induce a T cell proliferative response is an indicator of the helper capacity of a peptide epitope. We sought to verify this by measuring the capacity of the 965.10 peptide to deliver help in the generation of a CTL response. The CTL induction experiments were carried out according to the protocol provided below.

A. CTL induction protocol

Groups of three to six C57 BL/6J mice were immunized by subcutaneous injection at the base of the tail with a mixture of CTL and helper epitopes dissolved in PBS/5% DMSO and emulsified in IFA (Difco, Detroit, MI). After 11 days, mice were sacrificed and splenocytes prepared. Splenocytes ( $3 \times 10^7/10$  ml/T25 flask) were simulated in vitro by the addition of peptide-coated syngeneic lipopolysaccharide (LPS) blasts. LPS blasts were prepared 72 hr prior to use from splenocytes of C57BL/6J mice resuspended in medium containing LPS (W E coli 055:B5) (Difco, Detroit, MI) ( $25 \mu\text{g/ml}$ ), and Dextran Sulfate (Pharmacia, Uppsala Sweden) ( $7 \mu\text{g/ml}$ ). Cultures were prepared with  $1.5 \times 10^6$  splenocytes/ml in a total volume of 3 ml and incubated for 72 hr at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  in T75 flasks.

Subsequently, cells were irradiated, washed, and resuspended at  $30\text{--}40 \times 10^6$  cells/ml. One ml aliquots of this suspension were incubated with the CTL epitope at  $100 \mu\text{g/ml}$  for 1 hr at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . Cells were washed once and then resuspended at  $10 \times 10^6$  cells/ml. A volume of 1 ml was added per flask to the appropriate effector cells and incubated for 6 days. Cytotoxicity was then measured using EL4 (b haplotype) target cells ( $3 \times 10^6$  cells/ml) which were incubated at  $37^\circ\text{C}$  in the presence of sodium  $^{51}\text{Cr}$  chromate and CTL epitope peptide. After 60 min, cells were washed three times and resuspended in RPMI-1640 (Bio Whittaker, Walkersville, MD) containing 10% FCS (Irvine Scientific, Santa Ana, CA), 2mM L-Glutamine (Irvine Scientific),  $50 \mu\text{g/ml}$  Gentamicin (Irvine Scientific), and  $5 \times 10^{-5}$  M Beta Mercaptoethanol (Sigma, St. Louis, MO). Subsequently,  $1 \times 10^4$   $^{51}\text{Cr}$  labeled target cells were added to a titration of effector cells in U-bottom 96-well plates, final volume=200  $\mu\text{l}$ . After 6 hr incubation at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , 0.1 ml aliquots of supernatant were removed from each well and radioactivity determined in a Micromedic gamma counter. The percent specific lysis was determined by the following formula: % specific lysis =  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . Data are expressed in lytic units/ $10^6$  effector cells. One lytic unit

is arbitrarily defined as number of lymphocytes required to achieve 30% lysis of  $1 \times 10^4$   $^{51}\text{Cr}$ -labeled target cells within 6 hr, in absence or presence of peptide.

5                   We immunized C57BL/6J mice with a 10 nm dose of the  $\text{K}^b$  restricted (Carbonan and Bevan, J. Exp. Med. 169:603-612 (1989)) lipidated CTL determinant (Ova 257-264), along with varying amounts of the  $\text{IA}^b$  restricted helper epitopes Ova 323-336 (Wall et al., J. Immunol. 152:4526-4536 (1994)), HBV  
10 core 128-140, or the 965.10 peptide. After 11 days, spleen cells were stimulated in vitro with the CTL epitope Ova 257-264, incubated for 6 days, and then tested in a standard 6 hr Chromium release CTL assay. The CTL targets included both Ova 257-264 pulsed EL4 cells and Ova 257-264 transfected EG7  
15 cells.

#### B. Results

The results obtained are shown in Table V. It was found that the Pan DR epitope 965.10 induced a CTL response in  
20 a dose-dependent fashion, with an optimum of 307 lytic units observed when 5  $\mu\text{g}/\text{mouse}$  of 965.10 peptide was coinjected with the Ova 257-264 CTL epitope. In contrast, the helper activity of the Ova 323-336 and HBVc 128-140 epitopes was much less pronounced, both in terms of the magnitude of the helper  
25 effect (four-fold and three-fold increase, respectively) and of the dose required for induction of optimal helper activity (100  $\mu\text{g}/\text{mouse}$ ).

The above examples are provided to illustrate the  
30 invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

TABLE V: Helper Activity of Various Peptide Epitopes for CTL Induction In Vivo

T HELPER PEPTIDE	OPTIMAL DOSE OF HELPER PEPTIDE ( $\mu$ g/mouse)	CTL RESPONSE ( $\Delta$ LYTIC UNITS/EG CELLS)
---	---	12 $\pm$ 2
OVA 323-336	100	50 $\pm$ 5
HBV core 128-140	100	35 $\pm$ 10
965.10	5	307 $\pm$ 55

Specific lysis was calculated as described in Example 10.  
A representative of two independent experiments is shown.

WHAT IS CLAIMED IS:

1. A composition comprising a peptide consisting of 2 D-amino acids and 11 L-amino acids, said peptide having the formula  $R_1-R_2-R_3-R_4-R_5$ , proceeding from the amino-terminus to the carboxy-terminus, wherein:
- 5  $R_1$  is a D-amino acid followed by alanine or lysine;  
 $R_2$  is selected from the group consisting of cyclohexylalanine, tyrosine, and phenylalanine;  
 $R_3$  is 3 or 4 amino acids, wherein each amino acid is independently selected from the group consisting of alanine, isoleucine, serine and valine;  
10  $R_4$  is selected from the group consisting of threonine-leucine-lysine, lysine-threonine, and tryptophan-threonine-leucine-lysine; and  
15  $R_5$  consists of 2 or 4 amino acids followed by a D-amino acid, wherein each of the 2 or 4 amino acids is independently selected from the group consisting of alanine, serine and valine.
- 20 2. The composition of claim 1 wherein:  
 $R_1$  is D-alanine followed by alanine or lysine;  
 $R_2$  is cyclohexylalanine or phenylalanine;  
 $R_3$  is 3 or 4 amino acid, wherein each of the 3 or 4 amino acids is selected from the group comprising alanine, isoleucine, and valine; and  
25  $R_5$  is 2 or 4 alanines followed by D-alanine.
3. The composition of claim 2 wherein the peptide is selected from the group consisting of aAXAAAKTAAAAa, aAXAAAATLKAAa, aAXVAAATLKAAa, aAXIAAATLKAAa, aKXVAAWTLKAAa, and aKFVAAWTLKAAa wherein a is D-alanine, A is alanine, X is cyclohexylalanine, K is lysine, T is threonine, L is leucine, V is valine, I is isoleucine, W is tryptophan, and F is phenylalanine.  
35
4. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the peptide of claim 1.

5. A composition comprising a CTL inducing peptide and a T helper peptide, wherein the T helper peptide is a peptide of claim 1.

5 6. The composition of claim 5, wherein the CTL inducing peptide is acetylated, palmitylated, or acylated with a fatty acid.

10 7. The composition of claim 5, wherein the CTL inducing peptide is linked to the T helper peptide to form a CTL/T helper peptide conjugate.

8. The composition of claim 7, wherein the CTL/T helper peptide conjugate is linked to a carrier.

15 9. The composition of claim 6, wherein the CTL inducing peptide is linked to the T helper peptide by a spacer molecule.

20 10. The composition of claim 9, wherein the spacer is Ala-Ala-Ala.

25 11. A method of inhibiting activation of T cells in a patient, the method comprising administering to the patient a therapeutically effective dose of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a peptide of between about 4 and about 20 residues, the peptide being capable of binding antigen binding sites on MHC molecules encoded by substantially all alleles of a DR locus.

30 12. The method of claim 11 wherein the peptide is the peptide of claim 1.

35 13. The method of claim 11 wherein the peptide is the peptide of claim 3.

14. A method of inducing activation of T cell clones in a patient, the method comprising administering to the patient

a therapeutically effective dose of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a peptide of between about 4 and about 20 residues, the peptide being capable of binding antigen binding sites on MHC molecules encoded by substantially all alleles of a DR locus.

15  
16. The method of claim 14 wherein the peptide is conjugated to a CTL inducing peptide.

10  
17. The method of claim 14 wherein the peptide is the peptide of claim 1.

15  
18. The method of claim 14 wherein the peptide is is the peptide of claim 3.

FIGURE 1C

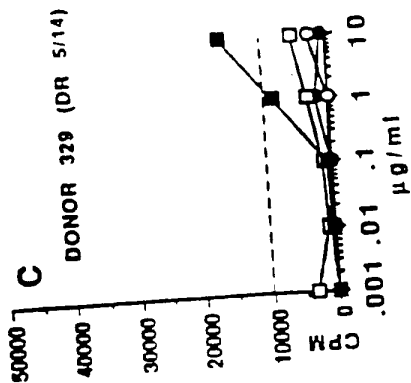


FIGURE 1B

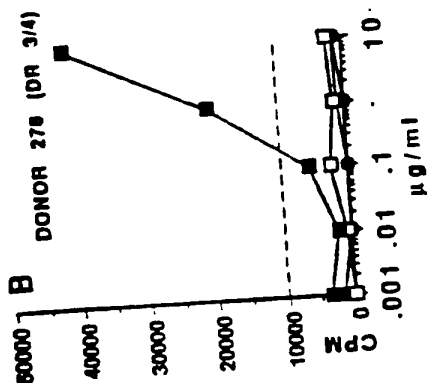


FIGURE 1A

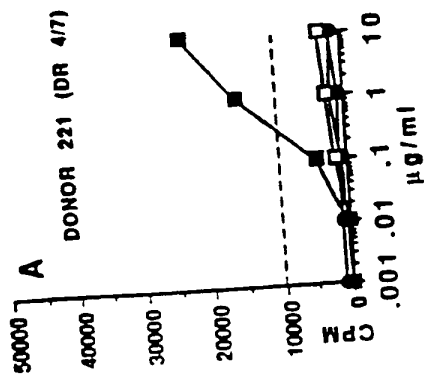


FIGURE 1F

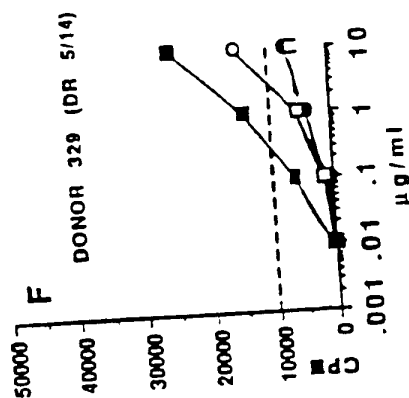


FIGURE 1E

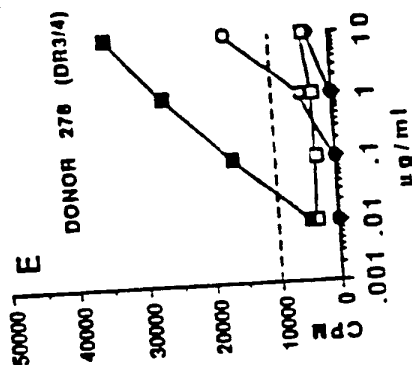


FIGURE 1D

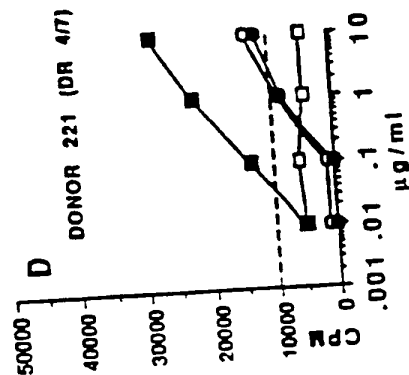




FIGURE 2A

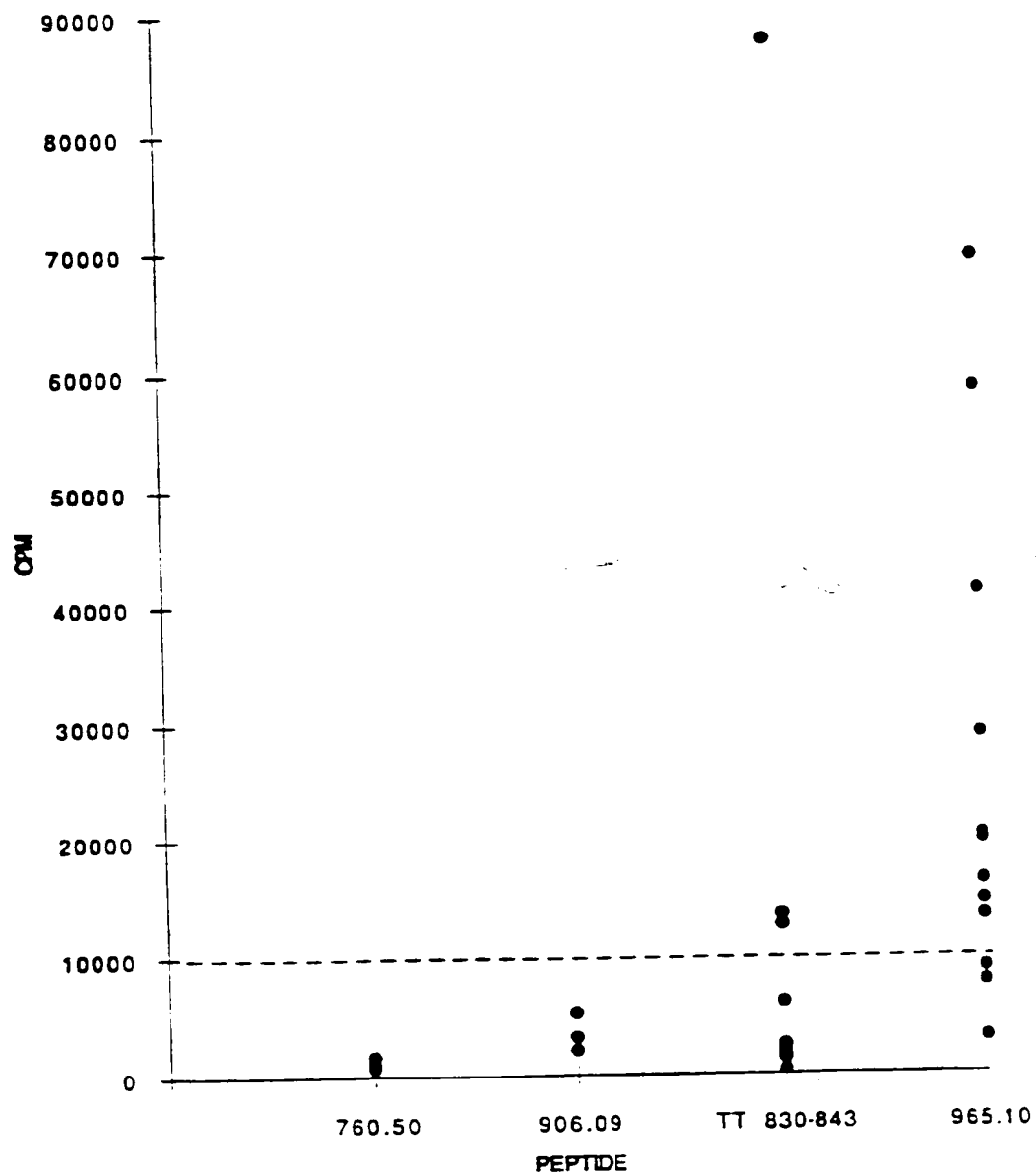


FIGURE 2B

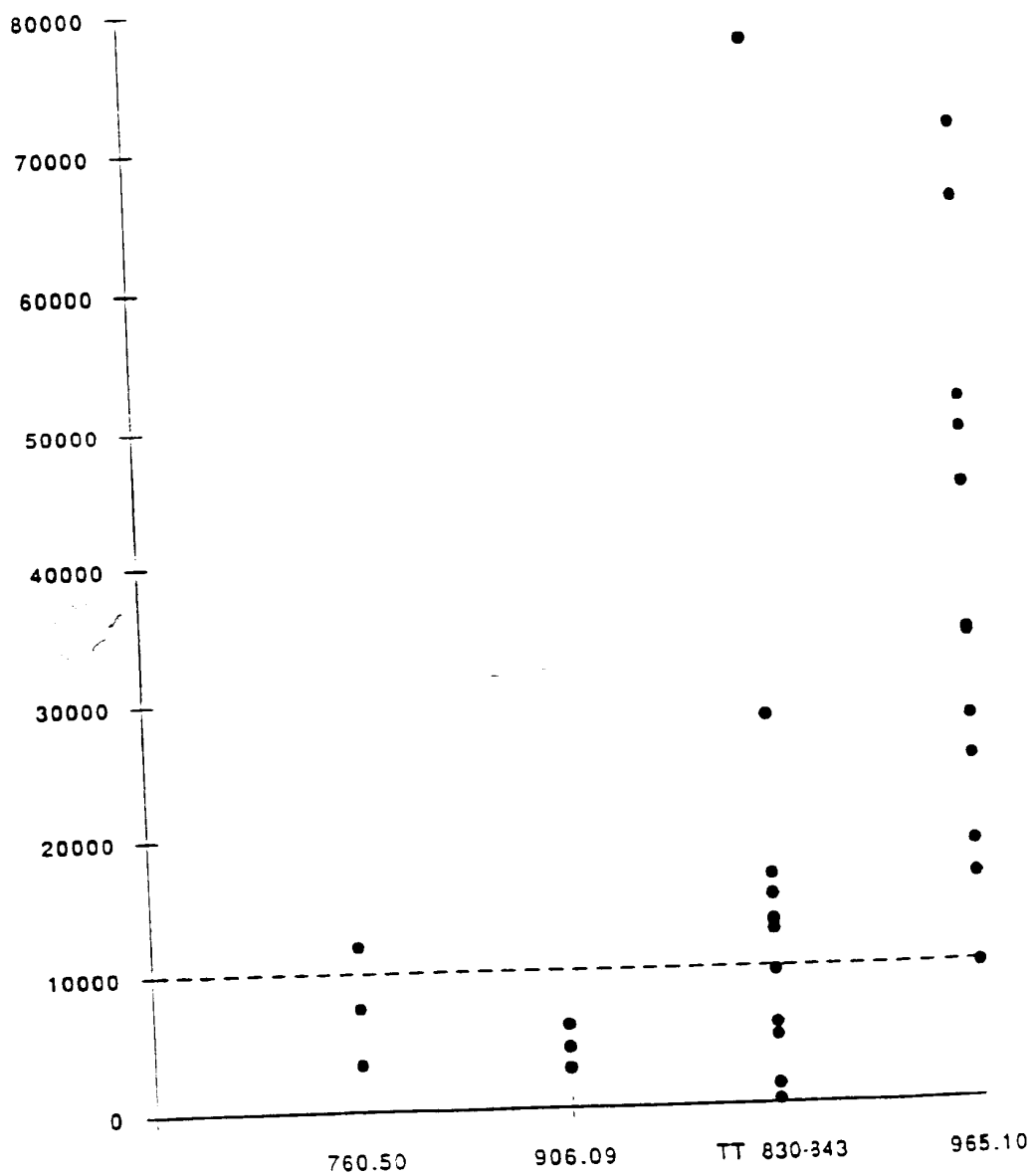


FIGURE 3A

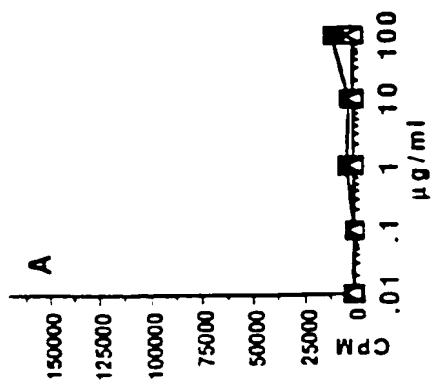


FIGURE 3B

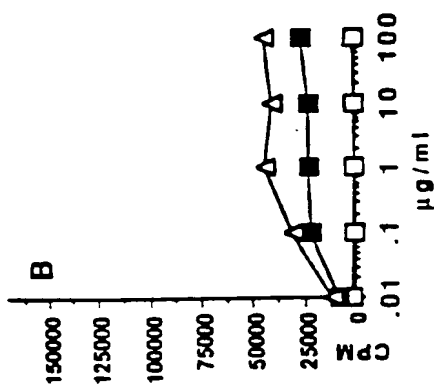


FIGURE 3C

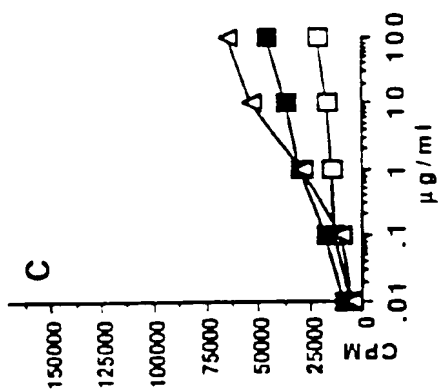


FIGURE 3D

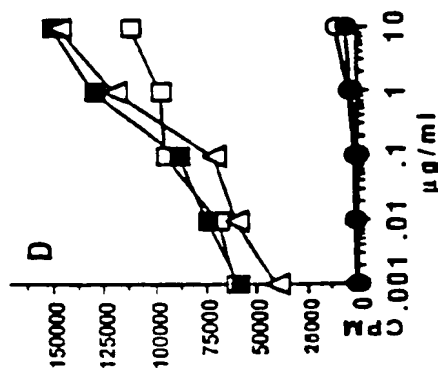


FIGURE 3E

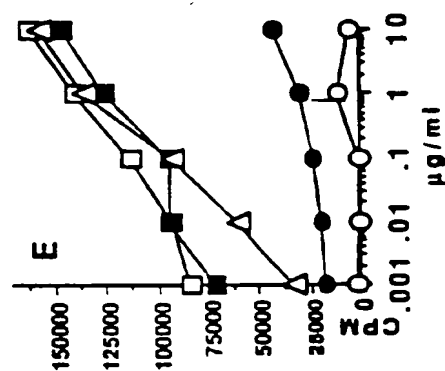
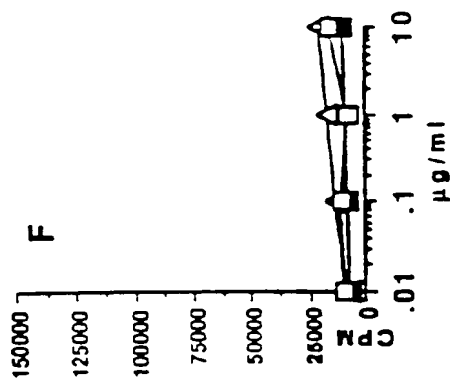


FIGURE 3F



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/10368

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/00; C07K 1/00, 5/00, 7/00, 14/00, 17/00.

US CL : 514/12, 14, 15, 885, 825, 903; 530/324,326, 327, 403, 812, 868.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 14, 15, 885, 825, 903; 530/324,326, 327, 403, 812, 868.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	US, A, 5,336,758 (BERZOFKY ET AL.) 09 AUGUST 1994, SEE ENTIRE DOCUMENT.	1-17
Y	US, A, 5,196,512 (BIANCHI ET AL.) 23 MARCH 1993, SEE ENTIRE DOCUMENT.	1-17
Y	US, A, 5,114,713 (SINIGAGLIA) 19 MAY 1992, SEE ENTIRE DOCUMENT.	1-17

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 DECEMBER 1994

Date of mailing of the international search report

09 JAN 1995

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

AVIS DAVENPORT

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

## Schering Research Plan – appendix B

A biological-based program developing GDF-8 inhibitors, in particular, vaccines capable of suppressing or inhibiting GDF-8 activity, seems to be the most viable way for promoting growth in food animals such as poultry, swine and cattle. M&E's proprietary AutoVac™ technology breaks immunologic tolerance to self-proteins and provides a simple and effective method for active immunization of non-immunogenic proteins.

M&E has designed 10 separate GDF-8 AutoVac™ constructs. The relatively high number of inter- and intra- molecular disulfide-bonds in the 109 amino acid residue C-terminal active region of GDF-8 mandates this large number. It is expected that not all of the constructs will elicit anti-GDF-8 antibodies in the target species. Syntro will make the genes for the constructs and incorporate the new genes in swinepox and avian herpes vector systems for further work. As an initial screening tool, naked DNA vaccine technology will be evaluated for its ability to identify potential candidate(s).

Two approaches to a GDF-8 autovaccine are currently being considered. The first approach is a recombinant subunit protein expressed from swinepox virus. In this approach the GDF-8 autoantigen is expressed in swinepox virus (SPV), purified and formulated with adjuvant as with a conventional killed vaccine. In the second approach, GDF-8 autoantigen is vectored in a modified live virus vaccine. In this approach a recombinant virus delivers the GDF-8 autoantigen. It will be manufactured and formulated similar to a conventional modified live vaccine.

*In vivo* studies in swine, cattle, or poultry monitoring parameters such as growth rate, muscle mass, and antibody responses will be used to identify the best GDF-8 vaccine candidate(s) and demonstrate proof of efficacy (PE). Similar studies could be conducted concurrently in mice using the various subunit vaccine candidates.

The steps delineated in the template below identify the key points in a generic R&D plan to implement the M&E AutoVac™ technology for generating a pharmaccine against any molecular target.

### Template research plan for AutoVac vaccine development (The sequence and timing to be specified as applicable for each project)

Task sequence (overlaps occur)	Comments
Target selection	
Gantt plan completed, incl. time & resource estimate (1 months)	Completion constitutes "beginning of research plan" re: 3.5.2
Molecule design (1-2 months)	
Synthesis genes (2-3 months)	
Analytical tools development (4-6 months)	
Selection of delivery system (1-3 months)	
Molecule expression (3-6 months)	
Molecule purification (3-6 months)	
Molecule characterisation (1-2 months)	
Demonstrate antigenic response in model or target animal (3-4 months)	



Demonstrate PE in target animal (4-6 months)	<ul style="list-style-type: none"> <li>a. Elicits one-off milestone payment to M&amp;E</li> <li>b. For first product: Starts period to "beginning of research plan" re: 3.5.2</li> <li>c. Potential license to M&amp;E for human use re: 3.1.4</li> </ul>
Select candidates for clinical development (1 month)	
Approval of development plan (1 month)	Elicits milestone payment to M&E for Additional Product re: 4.2
Availability of GMP clinical material (6-12 months)	
Trials conducted in target animal (24-36 months)	
Regulatory submission (1-3 months)	Elicits milestone payment to M&E re 4.2
Regulatory approval (24 months)	Elicits milestone payment to M&E re 4.2
First year sales completed	Elicits profit sharing payment to M&E re 4.2
Third year sales completed	Final product specific milestone calculation to M&E re: 4.2

